Plexiform-like lesions and increased tissue factor expression in a rat model of severe pulmonary arterial hypertension

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In advanced PAH of many etiologies, endothelial proliferation and medial hypertrophy ultimately obliterate the arterial lumen. Most patients also have disorganized cellular proliferation in glomeruloid structures called plexiform lesions (18, 23, 33). Plexiform lesions are not seen in systemic arterial disease; however, these unique structures resemble vessels in a rare form of cancer, glioblastoma multiforme (33). Although the role of plexiform lesions in the progression of PAH is controversial, one study of human lung specimens supports the hypothesis that plexiform lesions precede the development of concentric luminal obliteration (2). Because tissue from human patients with early-stage PAH is rarely available, an animal model that has distal smooth muscle hypertrophy, concentric luminal obliteration, and proliferative vascular lesions would be desirable to study the early changes and subsequent progression of PAH.

The endothelial toxin monocrotaline (MCT) is commonly used to produce an experimental model of moderate PAH in which animals develop endothelial damage and vascular smooth muscle hypertrophy. In another rat model, MCT is administered following left pneumonectomy. These animals develop more severe PAH with neointimal formation in most of the distal pulmonary vessels (21), similar to human disease. Although these models have been useful in defining pathways of vascular remodeling and thus suggesting novel treatments (4, 20, 35), the absence of plexiform lesions may be an important limitation. Plexiform lesions have not been consistently reported in rodent models of PAH (but see DISCUSSION).

To better understand the relationship between plexiform lesions and disease progression, we administered MCT to young rats following pneumonectomy, hypothesizing that younger animals would have a greater proliferative response to injury. We observed severe hemodynamic alterations typical of human disease and mortality earlier than that previously reported in rat models (6, 21). This more severe phenotype was associated with plexiform-like lesions and obliteration of the pulmonary arteriole lumens. Using a recently developed three-dimensional angiography technique, we found severe vascular pruning and networks of disorganized capillaries. We hypothesize that early vascular cell proliferation results in the more severe hemodynamic changes and early mortality that we observed.

PULMONARY ARTERIAL HYPERTENSION (PAH) is the marked elevation of precapillary resistance in the pulmonary circulation. It occurs in idiopathic form and in association with such diseases as congenital heart malformation, scleroderma, human immunodeficiency virus (HIV) infection, and cirrhosis (5, 7). There are few effective therapies for PAH, and even with the best available therapy, only 60% of patients live 5 yr, a striking statistic for patients whose mean age is less than 50 (14). While some progress has been made in treating patients, our understanding of the disease pathogenesis remains limited (19).
We then used this rat model to examine the expression of a molecule thought to influence vascular cell proliferation. Tissue factor (TF) is a transmembrane glycoprotein that initiates the coagulation cascade and may also facilitate angiogenesis, both in development and in tumor growth (15, 28). TF also mediates the smooth muscle proliferation leading to intimal hyperplasia following injury in the systemic circulation (24), and TF inhibitors have been useful in attenuating neointimal formation in animal models (31). Because PAH is a disease with neointimal formation (18, 23), disordered angiogenesis (2, 33), and a thrombotic diathesis (18, 23), we hypothesized that TF might be abnormally expressed in PAH. TF antigen was markedly increased in the vessels and plexiform-like lesions of these rats. Intense TF staining of diseased vessels was also seen in the lungs of humans with PAH. As is true with systemic arterial injury (31), induction of TF may contribute to the progression of PAH. Some of this work was presented in poster format at the Aspen Lung Conference in 2004 (34).

METHODS

Human subjects. Human tissue was obtained from University of Colorado PAH patients at autopsy (5) or at lung transplant (5). Informed consent to use the tissue for research purposes had previously been obtained. Unused donor lungs without evidence for pulmonary disease served as controls (6 subjects). Five patients with idiopathic PAH and five with associated PAH (scleroderma or HIV) were studied.

Animals. All animal studies were approved by the Institutional Animal Care and Use Committee at the University of Rochester before initiation. Male Sprague-Dawley rats weighing 200 g underwent pneumonectomy or sham surgery.

One week later, animals were given 60 mg/kg MCT (dissolved 60 mg/ml in DMSO) as a subcutaneous injection (vehicle animals received the same volume of DMSO). At the time of death (detailed in RESULTS), rats were anesthetized and given a percutaneous tracheostomy. Hemodynamics were measured in the open chest by direct puncture of the right ventricle with a fluid-filled catheter utilizing a Power Lab apparatus connected to a Macintosh computer. Analog signals were digitized at 100 Hz, and 10–60 s of reproducible cardiac cycles were recorded from the right ventricle and then the main pulmonary artery (PA). The left atrium was cut, and the lungs were perfused with phosphate-buffered saline. Fluorescein microspheres (0.2 μm; Molecular Probes, Eugene, OR) were suspended in 1% low-melting point agarose (10% microspheres in final volume 1% agarose) and injected into the PA (35). The lungs were inflation fixed using a 20-cm column with 4% paraformaldehyde. The heart chambers were washed. After 24–72 h of paraformaldehyde, the heart chambers were transferred to 70% ethanol. Right lower lobe tissue was blocked using a 20-cm column with 4% paraformaldehyde. The heart chambers were washed. After 24–72 h of paraformaldehyde, the lungs were transferred to 70% ethanol. Right lower lobe tissue was blocked and paraffin embedded.

Histopathology. Rat and human sections were cut at 5 μm, deparaffinized, and rehydrated through graded alcohols. Staining for elastic lamina was performed using a combined elastin and modified trichrome stain. The slides were sequentially exposed to picric acid, Verhoeff’s elastic, and then 2% ferric chloride. Sections were evaluated for elastic lamina visibility before staining with Biebrich scarlet-acid fuschin, phosphotungstic acid, and fast green. The resulting sections show black elastic fibers, blue to black nuclei, and green collagen; all other tissue elements are purple to pink.

For immunohistochemistry, slides were quenched in 3% hydrogen peroxide and then antigen retrieved using a pressure cooker in 10 mM citrate buffer at pH 6. The slides were blocked in serum (Vector Laboratories, Burlingame, CA), and the primary antibody was applied overnight at 4°C. Immunodetection was performed with biotinylated secondary (Vector), peroxidase-labeled streptavidin (Jackson ImmunoResearch, West Grove, PA), and Nova Red (Vector). The slides were hematoxylin counterstained. The primary antibody was omitted as negative control with every group of slides. Vascular endothelial growth factor receptor-2 (VEGFR-2, flk-1) antibody was purchased from Santa Cruz, von Willebrand factor (vWF) was from Dako, and smooth muscle α-actin (SMA) was from Sigma (clone 1A4). TF immunohistochemistry for rat and human employed a polyclonal anti-human TF antibody raised in rabbits against the extracellular domain of TF (17), and the findings in rats were confirmed using a monoclonal anti-rat TF monoclonal antibody (clone SD12) generously provided by Daniel Kirchhofer at Genentech (San Francisco, CA). No staining was observed when secondary antibody was omitted (an additional negative control).

Fluorescence microangiography images were made using a Leica laser scanning confocal microscopy system. Two-hundred-micrometer sections of fixed lung were cut on a vibratome, and images were obtained on an upright microscope using standard fluorescence settings. To determine the extent of vascular perfusion (see Fig. 5, A and B), three separate lung slices were imaged at low power (4 mm²), and images of three regions from each lung slice were obtained for each rat. The tissue depth, laser intensity, photomultiplier gain, and optical slice thickness (~50 μm) were standardized for all images. Although true blinding was not possible, bias was avoided by capturing three images per slice covering ~40% of the total slice area. For high-resolution images (Fig. 5, C–E), large vessels were identified through the eyepiece with a conventional Hg lamp, and then 40–80 z-section images were obtained to cover the entire network associated with a large vessel (total depth 60–100 μm). There was no evidence of photobleaching. Three-dimensional reconstructions were made utilizing NIH Image J with a ‘‘brightest point’’ algorithm and standard opacity at 20%.

Statistical analysis. Hemodynamic data was analyzed with the Mac Lab software to generate the summary data shown in Fig. 1. Representative tracings (at least 4 cardiac cycles) were highlighted; the computer software then generated means and peak pressures. The four groups in Fig. 1 A and B, and Table 1 were first compared using an analysis of variance with an α-level of 0.05 followed by post hoc testing using Tukey’s HSD test (R, R Foundation for Statistical Computing, Vienna, Austria). In accord with APS standards, data are expressed as means ± SD.

RESULTS

Severe PAH develops after MCT administration to pneumonectomized rats. Young rats (200 g, ∼6 wk old) were subjected to a left pneumonectomy or sham surgery. One week later, they were given a single dose of MCT (60 mg/kg) or vehicle injection. The initial reports using older rats showed data from animals killed 28 days following MCT administration (6, 21), and we planned identical experiments. However, in the group of animals for Fig. 1 A, we had two spontaneous deaths before day 24; therefore, the remaining rats treated with pneumonectomy plus MCT were killed at day 24. Figure 1A shows that these rats had severely elevated mean PA pressures compared with rats treated with MCT following a sham surgery. Interestingly, pneumonectomy plus vehicle had no effect on mean PA pressure compared with sham-operated, vehicle-treated
animals. Because the septum becomes a functional part of the RV in severe PAH, we calculated RV hypertrophy as (RV + septum)/left ventricular weight. Table 1 demonstrates that RV hypertrophy was increased in animals treated with pneumonectomy plus MCT compared with sham plus MCT ($P < 0.03$).

In light of the early deaths, subsequent groups of animals were euthanized at 21 days. Figure 1A shows that pneumonectomy plus MCT-treated rats developed marked PAH at this early time point, whereas sham plus MCT animals had relatively normal hemodynamics. RV hypertrophy in pneumonectomy plus MCT was similar to that at 28 days (data not shown). In a subsequent experiment, we implanted telemetry probes to monitor daily the progression of hemodynamic changes. An example tracing of RV pressure from an awake behaving rat is shown in Fig. 1C. We found substantial daily variation in the absolute values of RV systolic and diastolic pressure in some of the control animals that did not receive MCT (data not shown). However, the RV pulse pressure was consistent day to day, and thus we utilized this number to monitor the development of PAH (Fig. 1D). Compared with a recent report of daily hemodynamic monitoring after MCT alone (29), pneumonectomy plus MCT rats developed substantial increases in RV pulse pressure beginning 8 days after MCT and had pulse pressures in excess of 75 mmHg by day 12 after MCT (Fig. 1D). We observed a 30–40 mmHg drop in right ventricular systolic pressure during general anesthesia induction in animals with telemetry pressure probes (data not shown), and we therefore think that the increased pressures in PAH rats from Fig. 1, A and B, are substantially underestimated because of general anesthesia.

Severe PAH rats have histopathology similar to human disease. Pneumonectomy animals treated with vehicle had normal two-dimensional vascular histology (Fig. 2, A and D). Pneumonectomy plus MCT-treated rats had pulmonary arte-
In contrast, sham plus MCT animals (Fig. 2, treated with MCT. Scale bar in vehicle. A: low-power view of lung from a pneumonectomy animal treated with elastin to delineate the intima and media in small pulmonary arterioles. and smooth muscle hypertrophy. Rat lung tissue was stained with trichrome to 305x722 ing sham surgery or pneumonectomy (Figs. 2, A). A distinctive finding in our study was the high frequency of perivascular, proliferative lesions (Figs. 3, D–G). Consistent with previous reports (6, 21), we performed immunostaining in serial sections to characterize the cellular phenotypes in the plexiform-like lesions from rats, with antibodies to VEGFR-2 and VWF. The rat plexiform-like lesions contained mature, vWF-expressing endothelial cells (Fig. 3D) lining vascular channels, whereas endothelial cells staining for VEGFR-2 (but lacking vWF) were more uniformly distributed (Fig. 3E). We also observed SMA antigen in the plexiform-like lesions (data not shown) suggesting that, just as with human plexiform lesions, both endothelial cells (EC) and smooth muscle cells (SMC) contribute to the disease process. In summary, this model recapitulates key pathological features (neointimal formation, smooth muscle hypertrophy, and plexiform-like lesions) of human idiopathic PAH.

To explore our hypothesis that plexiform-like lesions are an early part of the disease progression and not an artifact of end-stage disease, we performed pneumonectomies on a group of 16 rats and then killed cohorts 1 wk later (day 0, the day other rats received MCT) and then at 7, 14, and 21 days after MCT. Serial sections of tissue from these animals were stained for elastin and SMA. One week following pneumonectomy, the contralateral lung had normal alveolar histology (Fig. 4A), and the small elastin(+) arteries had no SMA staining (Fig. 4C). Seven days after MCT, there were many normal arteries (Fig. 4, D and F), but occasional plexiform-like lesions with perivascular proliferation were also observed. At day 14, arteries with concentric SMA(+) muscular hypertrophy (Fig. 4, G and I) were observed, and many of these arteries were already partially or completely occluded by cells that did not stain for SMA. By day 21, most arteries had SMA(+) muscular hypertrophy and were at least partially occluded (Fig. 4, J and L).

Similar to the classic description of human plexiform lesions occurring distal to branch points (9) and human autopsy reconstructions (2), we occasionally observed a plexiform-like lesion with perivascular proliferation at a clear branch point (Fig. 4, J–L, p*). With the progressive vascular changes, there appeared to be a corresponding decrease in the density of alveolar epithelial tissue (compare Fig. 4A with 4J).

Severe PAH rats have vascular pruning and proliferation of disorganized vascular networks. A novel technique for fluorescence microangiography was recently described to examine the lung vasculature of rats and mice (11, 35) in which a suspension of fluorescent microparticles with the viscosity of blood is flushed through the lungs during death. We utilized this technique to complement the standard histopathology and characterize the density of capillary networks in 200-μm-sections of fixed lung tissue. Figure 5A shows a representative 50-μm “optical slice” of rat lung 1 wk following a pneumonectomy; most of the field is filled with fluorescent vessels. In contrast, Fig. 5B illustrates a distinct pruning of the vascular

Fig. 2. Rats treated with MCT following pneumonectomy develop neointima and smooth muscle hypertrophy. Rat lung tissue was stained with trichrome to illustrate normal alveolar epithelium and several perivascular collections of cells with a plexiform-like appearance. Scale bar indicates 100 μm. A–C: ×40 views of the vessels marked with arrows in A–C. D: ×40 view of an ~60-μm arteriole marked with arrow from A; note the thin smooth muscle layer even in this larger vessel. E: destruction of the elastic lamina (arrow) and extensive neointima of a 30-μm vessel from B. F: a different vessel from the same field with smooth muscle hypertrophy but no neointima (arrow indicates intact elastic lamina). G: illustrates that sham-operated MCT animals often had smooth muscle hypertrophy but never had the neointimal changes shown in E (intact elastic lamina marked by arrow). Micrographs are representative of 8 pneumonectomy + vehicle animals, 30 pneumonectomy + MCT rats, and 12 sham-operated MCT rats.

Fig. 3. Plexiform-like lesions in rats have endothelial cells staining for both von Willebrand factor (vWF) and vascular endothelial growth factor receptor (VEGFR)-2 (2). To characterize the cellular phenotypes in the plexiform-like lesions from rats, we performed immunostaining in serial sections with antibodies to VEGFR-2 and vWF. The rat plexiform-like lesions contained mature, vWF-expressing endothelial cells (Fig. 3D) lining vascular channels, whereas endothelial cells staining for VEGFR-2 (but lacking vWF) were more uniformly distributed (Fig. 3E). We also observed SMA antigen in the plexiform-like lesions (data not shown) suggesting that, just as with human plexiform lesions, both endothelial cells (EC) and smooth muscle cells (SMC) contribute to the disease process. In summary, this model recapitulates key pathological features (neointimal formation, smooth muscle hypertrophy, and plexiform-like lesions) of human idiopathic PAH.

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network, and large areas of the optical slice have no fluorescence in a pneumonectomy plus MCT rat at day 21. We also observed collections of bright fluorescence (marked by arrows in Fig. 5B) that we believe correspond to the plexiform-like lesions in Figs. 3 and 4.

To obtain more detailed images of the normal and diseased pulmonary vasculature, we obtained z-series confocal images at higher resolution (supplemental material for this article is available online at the AJP-Lung web site, which shows the complete z-stack as a movie) and made stereo projections of the rat lung vasculature (Fig. 5, C–E). The normal rat lung has a network of capillaries that are uniformly distributed over the field of view (Fig. 5C and online movie), and at the highest magnification, the vessels have a fine character (Fig. 5C, inset). In contrast, high-resolution reconstructions of rat vasculature at day 7 after MCT (Fig. 5D) and day 21 after MCT (Fig. 5E) confirm the extensive vascular pruning observed at lower power. We consistently observed unusual appearing vessels at the highest magnification (Fig. 5D, inset) that appeared to be the three-dimensional correlate of the plexiform-like lesions seen in Figs. 3 and 4. In addition, rats with severe PAH (pneumonectomy + MCT) also had severe luminal narrowing in the large pulmonary trunks (Fig. 5E).

Fig. 4. Plexiform-like lesions and tissue factor (TF) expression both develop early in the disease evolution. Rats were killed before MCT (A–C) and at day 7 (D–F), day 14 (G–I), and day 21 (J–L) after MCT. Serial sections from 3–4 rats at each time point were stained for elastin fibers (A, D, G, and J), TF antigen (B, E, H, and K), and smooth muscle α-actin (SMA; C, F, I, and L). Arrows in A–F show a non-muscularized pulmonary arteriole ~30–60 μm before MCT (A–C) and 2 arterioles in a rat with early disease (D–F). Arrows in G–L show similar-sized arterioles with concentric medial hypertrophy, SMA staining, and partial (J–L) or complete (G–I) luminal obliteration. Bronchi are denoted with a b as a positive control for both TF and SMA staining. Plexiform-like lesions are denoted with a p, and p* denotes a plexiform-like lesion at an apparent branch point. Note that vascular TF antigen expression occurs early (E) and progresses in intensity from day 14 to 21. Intense SMA staining occurs later (days 14 and 21). The serial sections confirm the impression of disorganized vascular channels in plexiform-like lesions.

TF is induced in the vessels and plexiform-like lesions of rats treated with pneumonectomy and MCT and in vascular lesions of patients with PAH. Rats undergoing pneumonectomy followed by vehicle treatment had very little medial TF staining (Fig. 4B). Because we hypothesized that TF induction would occur early and contribute to vascular cell proliferation, the development of plexiform-like lesions, and luminal obliteration, we examined TF staining at days 7, 14, and 21 following MCT exposure. Morphologically normal vessels at day 7 had little TF staining (Fig. 4E, arrows), whereas TF staining was clearly present in the cells of a plexiform-like lesion (Fig. 4E, p). TF staining was intense in most vessels and in the plexiform-like lesions at days 14 and 21 (Fig. 4, H and K).

To confirm this finding in human disease, lung tissue was obtained from PAH patients who were undergoing transplant or at autopsy at the University of Colorado Pulmonary Hypertension Center (see Methods for patient characteristics). TF was rarely seen in the media or endothelium of vessels from normal patients (Fig. 6A). In contrast, patients with PAH had prominent medial TF staining even in vessels with relatively early abnormalities (Fig. 6B). TF staining was heavy in vessels that were obliterated by typical concentric proliferation (Fig. 6C) and was particularly intense in many plexiform lesions.
The fine network of vessels distributed uniformly over the field in images with an optical slice thickness of ~50 μm from pneumonectomized rats before (A) or 21 days after (B) 60 mg/kg MCT. Scale bar shows 500 μm. Compare the fine network of vessels distributed uniformly over the field in A with the large areas in B that have little or no fluorescence (a, a large airway; no airways in B). Arrows in B denote bright collections of fluorescence never observed in pneumonectomized rats before MCT and seen often after MCT. C–E: stereo reconstructions of thin confocal images acquired at higher resolution to illustrate the capillary networks (scale bar, 100 μm). C: a branching precapillary pulmonary arteriole in a pneumonectomized rat; inset shows a fine capillary structure. D: early capillary loss 7 days after MCT. Note that the central vessel does not appear to be narrowed. The inset shows a vascular structure reminiscent of a hemangioma and is quite distinct from anything seen in control animals (arrow). E: a reconstruction from a 21-day rat; arrows mark the narrowed lumen (compare to lumens in Fig. 4J), and the capillary network is much less dense than in C. A and B are representative of 9 similar fields each from 3–4 different rats (see METHODS), and C–E represent at least 3 similarly examined vessels.

Fig. 5. Three-dimensional angiography reveals extensive vascular pruning and the development of disorganized vascular networks. At death, the PA was injected with fluorescein-conjugated microparticles, and confocal images of inflation-fixed rat lung were acquired as described in METHODS. A and B are single confocal images with an optical slice thickness of ~50 μm from pneumonectomized rats before (A) or 21 days after (B) 60 mg/kg MCT. Scale bar shows 500 μm. Compare the fine network of vessels distributed uniformly over the field in A with the large areas in B that have little or no fluorescence (a, a large airway; no airways in B). Arrows in B denote bright collections of fluorescence never observed in pneumonectomized rats before MCT and seen often after MCT. C–E: stereo reconstructions of thin confocal images acquired at higher resolution to illustrate the capillary networks (scale bar, 100 μm). C: a branching precapillary pulmonary arteriole in a pneumonectomized rat; inset shows a fine capillary structure. D: early capillary loss 7 days after MCT. Note that the central vessel does not appear to be narrowed. The inset shows a vascular structure reminiscent of a hemangioma and is quite distinct from anything seen in control animals (arrow). E: a reconstruction from a 21-day rat; arrows mark the narrowed lumen (compare to lumens in Fig. 4J), and the capillary network is much less dense than in C. A and B are representative of 9 similar fields each from 3–4 different rats (see METHODS), and C–E represent at least 3 similarly examined vessels.

Discussion

This is the first report of an experimental PAH model in which medial hypertrophy, neointimal formation with concentric luminal obliteration, and plexiform-like lesions are consistently observed. We subjected young rats to MCT 1 wk after pneumonectomy, and they developed a degree of PAH and RV hypertrophy much greater than those treated with MCT following sham operation, as previously reported (21). In addition, these younger rats died sooner than those in earlier reports and had very severe pruning of the vessels imaged by fluorescence microangiography. We therefore propose that this approach creates a severe PAH phenotype that may be a more relevant approximation of human disease than the rat MCT model most widely utilized.

The role that plexiform lesions play in the pathogenesis of human PAH is controversial. Human autopsy data have suggested that the lesions are an active part of the pathology and that they precede concentric luminal obliteration (2). In that report, plexiform lesions appeared at branch points. Computer-aided, three-dimensional reconstructions of two-dimensional histology suggested that actively dividing cells in plexiform lesions ultimately resulted in a concentric onion skin lesion and luminal occlusion immediately proximal to the branch point. To model this in rats, Taraseviciene-Stewart et al. (30) administered a VEGFR blocker to hypoxic rats and reported endothelial cell proliferation, luminal obliteration, and severe PAH. This alternative model of human PAH simulates the excess vascular cell proliferation, but they did not observe lesions that appeared to have vascular channels with vWF positive cells lining the channels (Figs. 3, 4, and 5). Greenway et al. (10) reported that overexpression of the S100A4/Mts1 protein in CBA × C57/B16 mice resulted in 5% of the mice developing plexiform-like lesions and neointimal changes. Because this protein is associated with metastatic potential and angiogenesis in human and mouse tumors, this was an exciting observation. However, the low prevalence (5%) of the lesions makes these mice less attractive as a model for studying PAH. Finally, Ivy and colleagues (13) reported severe PAH and clear neointimal lesions after administering MCT to rats genetically deficient in the endothelin-B receptor. Their animal model of severe PAH shares important histological features with ours: they observed destruction of the internal elastic lamina, the proliferation of EC and SMC, and severe vascular pruning as assessed by a barium gelatin angiography. However, they did not observe structures that appeared to proliferate outside the anatomic lumen nor did they observe structures that appeared to have vascular channels on serial sections. Their angiography technique also did not reveal structures consistent with disordered angiogenesis.

In the present study, rats with severe PAH have disorganized cellular proliferation in plexiform-like lesions that exhibit a visual similarity to human plexiform lesions. In addition, the pattern of endothelial cell staining is similar to that of human plexiform lesions (2). Immature endothelial cells expressing VEGFR-2 were found throughout the plexiform lesions, and mature endothelial cells expressing vWF lined the lumens of the vascular spaces. The disorganized cellular proliferation was a consistent finding that occurred early in disease progression (Fig. 4) and correlated with the development of neointimal changes. The early development of these lesions strongly suggests that these structures play a role in disease progression and are not an epiphenomenon of end-stage PAH. These rats...
had more severe hemodynamic alterations than that seen in rats treated with MCT alone, and we observed spontaneous deaths before day 28 (never observed with MCT alone.) The administration of MCT 1 wk following pneumonectomy has been used to model PAH in rats by other authors (20, 21). Previous investigators, however, used animals 350–400 g (~12 wk), whereas this study employed younger animals (200 g, 6 wk). Rats have a remarkable ability for lung proliferation following contralateral pneumonectomy (1); it may be that younger rats are more prone to have vascular cell proliferation in response to the combined stimulus of endothelial injury (MCT) and pneumonectomy. If so, this rat model may be a better tool than MCT alone for resolving controversies about the mechanisms that underlie vascular cell proliferation characteristic of the human disorder (2, 32, 33).

We have addressed one such controversy about the plexiform lesions that is difficult to answer in humans. The presence of prominent VEGF antigen staining and mRNA in endothelial cells has prompted some investigators to suggest that plexiform lesions are a form of disorganized angiogenesis (32). Implicit is the assumption that these plexiform lesions are connected to the pulmonary arterial circulation. In humans, it has not been possible to test this assumption directly. In our rats, low-power, three-dimensional angiography showed tufts of brightly staining vessels (Fig. 5B); at higher power, the fine reticular network in controls (Fig. 5C) contrasted with larger caliber globular vessels (Fig. 5D) bearing a resemblance to vascular tumors (like hemangiomas). Importantly, the size of the two-dimensional structures in Fig. 3 (~100 μm) correlates to the size of the structures in the microangiography pictures. Thus, this microangiography data provide evidence that the two-dimensional structures in Figs. 3 and 4 are connected to the pulmonary circulation. Zhao et al. (35) initially described this fluorescence technique for rats treated with MCT alone. They observed vascular pruning in MCT-treated animals, but they did not report the development of atypical vascular structures such as these (even at 35 days after MCT.) We propose that the plexiform-like lesions seen in two-dimensional histology correlate with the structures observed in the fluorescence microangiography and that these structures arise from the pulmonary arterial circulation.

TF is normally present at low levels in the intima and media of the arterial wall. The finding of increased TF expression in patients and rats with PAH suggests an explanation for the in situ thrombosis first observed in autopsy studies over 20 years ago (8). In that report of largely clinical data, in situ thrombosis was a major finding in the autopsies that were performed, despite the fact that chronic thromboembolic disease was excluded clinically. A second autopsy series examined specimens from patients in the NHLBI Pulmonary Hypertension Registry (23). In these 58 patients with idiopathic disease, 19 patients had thrombotic lesions. Recanalized thrombi were observed in 9/25 patients with plexiform lesions (23), demonstrating that in situ thrombosis and plexiform lesions coexist in some patients with idiopathic disease.

Clinical data also support the idea that PAH is a disease with a thrombotic diathesis (14, 27). The first report from Chicago was an examination of mortality in a prospective observational trial of calcium channel blockade (27). All patients given warfarin had perfusion lung scans that suggested “nonuniform” blood flow, but none met criteria to diagnose embolic disease. Warfarin use was associated with decreased mortality at 5 years, and warfarin therapy became the standard of care. A more recent report from Columbia Presbyterian observed consecutive patients from 1994 to 2002, including those treated with prostanoids (14). They also observed a survival benefit with warfarin. Increased TF expression at the arterial luminal surface would predispose patients to have in situ thrombosis. Our finding of increased TF expression in severe PAH thus suggests an explanation for this long-recognized prothrombotic tendency.

The increase in TF may play other roles in the pathogenesis of PAH. TF activation produces factor Xa and thrombin, both of which regulate intracellular signaling in vascular EC and SMC via protease-activated receptor (PAR) family members (3, 28). Thrombin increases endothelial permeability in conduit vessels and promotes SMC migration and proliferation (22) through interaction with its G protein-coupled PAR. PAR activation also mediates adhesion molecule expression and thus influences inflammatory cell recruitment (26). Because TF expression has been linked to angiogenesis (16) and the cellular proliferation following injury to the systemic circulation (25), increased TF activity might also mediate the disorganized angiogenesis and cellular proliferation seen in plexiform lesions.
In summary, we report a rat model of PAH with severe hemodynamic alterations, neointimal formation, and plexiform-like lesions. The lesions have a morphology and pattern of endothelial antigen staining similar to human plexiform lesions. Microangiography demonstrates severe vascular pruning and suggests angiogenesis leading to the development of disorganized vascular networks. We also found intense TF staining in the plexiform-like lesions and vessels of these rats and humans. The rat model will allow investigation of the signaling that generates plexiform lesions and should allow us to test the hypothesis that plexiform lesions precede and contribute to the development of severe disease. The model also allows investigators to test agents that might prevent lesions or even cause regression of established plexiform lesions. Proximal inhibitors of TF activity will reveal whether TF expression and activity is causally important in PAH.

REFERENCES


